Conditional Targeting of TRAF6 Reveals Opposing Functions of TLR Signaling in Endothelial and Myeloid Cells in a Mouse Model of Atherosclerosis

Running title: Polykratis et al.; TRAF6 in atherosclerosis

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Abstract:

**Background** - Previous studies implicated Toll-like receptor (TLR) signaling as a critical pathogenic pathway in atherosclerosis, but the cell-specific mechanisms by which TLRs act to control atherosclerotic plaque development remain poorly understood.

**Methods and Results** - To study the cell-specific role of TRAF6 in atherosclerosis, we generated *ApoE*−/− mice with endothelial cell-specific or myeloid cell-specific TRAF6 deficiency using Cre/LoxP-mediated gene targeting. Endothelial TRAF6 deficiency reduced atherosclerosis in female *ApoE*−/− mice by inhibiting NF-κB-dependent proinflammatory gene expression and monocyte adhesion to endothelial cells. In contrast, myeloid cell-specific TRAF6 deficiency caused exacerbated atherosclerosis with larger plaques containing more necrotic areas in both male and female *ApoE*−/− mice. TRAF6-deficient macrophages showed impaired expression of the anti-inflammatory and atheroprotective cytokine IL-10, elevated ER stress, increased sensitivity to oxLDL-induced apoptosis and a reduced capacity to clear apoptotic cells. Thus, the reduced anti-inflammatory properties coupled with increased sensitivity to apoptosis and impaired efferocytosis capacity of TRAF6-deficient macrophages result in exacerbated atherosclerosis development in TRAF6MYKO/*ApoE*−/− mice.

**Conclusions** - In conclusion, TLR-mediated TRAF6 signaling acts in endothelial cells to promote atherosclerosis, but displays atheroprotective anti-inflammatory and pro-survival functions in myeloid cells.

**Key words**: atherosclerosis; cytokines; endothelium; inflammation; macrophage
Introduction

Atherosclerosis is the major cause of death in westernized societies, being the underlying cause for cardiovascular diseases such as heart attack and stroke. Currently, atherosclerotic lesions are appreciated as areas of vessel wall inflammation, where complex interactions of modified lipoproteins, leukocytes (monocytes/macrophages, dendritic cells and lymphocytes) as well as vascular endothelial cells and smooth muscle cells take place. During the early stages of atherosclerosis oxidized lipids and pro-inflammatory cytokines activate endothelial cells to express chemokines and adhesion molecules, which induce the recruitment and transmigration of monocytes into the intima of large vessels. Inside the intima monocytes differentiate into macrophages and phagocytose lipids eventually becoming lipid-laden foam cells. Foam cells secrete cytokines and chemokines further inducing the recruitment of inflammatory cells and finally die in the lesions contributing to the formation of a necrotic core. The link between inflammation and atherosclerosis is strengthened by evidence that inflammatory diseases such as periodontitis or autoimmune diseases like lupus are associated with increased risk for atherosclerosis. Although the inflammatory nature of atherosclerosis is now widely accepted, the functions of specific components of the innate and adaptive immune response cascades in disease onset, progression and severity remain poorly understood.

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and induce intracellular signaling cascades regulating innate immune responses essential for host defense. In addition to PAMPs, TLRs also recognise endogenous ligands that are related to atherosclerosis such as modified LDL and HSP-60, suggesting that TLR-mediated responses may contribute to the pathogenesis of atherosclerosis. Indeed, both resident cells and recruited leukocytes within atherosclerotic plaques were shown to express TLR1, TLR2, TLR4 and...
TLR5⁴,⁸,⁹. Functional analysis of the role of TLRs in an in vitro model of human atherosclerosis suggested that TLR2 is the main receptor mediating inflammation and matrix degradation¹⁰. Moreover, increased expression of TLR2 in monocytes was recently identified as an important risk factor for atherosclerosis¹¹. The first direct in vivo evidence that TLR signaling is important for the pathogenesis of atherosclerosis came from the observation that ApoE⁻/⁻ mice lacking MyD88, an adapter protein required for signaling downstream of all TLRs except TLR3, are protected from diet-induced atherosclerosis¹². Furthermore, TLR4 deficiency was associated with a significant reduction of aortic plaque size, lipid content, and macrophage infiltration in ApoE⁻/⁻ mice¹³. However, MyD88 deficiency had a stronger impact in inhibiting atherosclerosis compared to the TLR4 knockout effect, suggesting that other TLRs signaling via MyD88 might also contribute to the development of atherosclerosis. Indeed, TLR2 gene ablation modestly reduced atherosclerosis severity in LDLR⁻/⁻ mice, an effect proposed to be mediated by non-hematopoietic cells of the vascular wall¹⁴. However, more recent experiments also provided evidence for a pro-atherogenic role of TLR2 in hematopoietic cells¹⁵. Furthermore, administration of a TLR2 agonist increased the severity of atherosclerosis in the ApoE⁻/⁻ mouse model, an effect dependent on TLR2 expression in bone-marrow-derived cells¹⁶. In contrast to the pro-atherogenic roles attributed to TLR2 and TLR4, a recent study showed that TLR3-deficiency accelerated the onset of atherosclerosis in ApoE⁻/⁻ mice¹⁷ suggesting that TLR3 exerts anti-atherogenic functions. While these studies established TLR signaling as a crucial regulator of atherosclerosis, the mechanisms by which TLRs contribute to the initiation and progression of atherosclerotic plaque development remain poorly understood. Elucidating the cell-specific function of TLR signaling in atherosclerotic plaque development will be essential for
understanding the mechanisms by which these receptors contribute to the pathogenesis of atherosclerosis.

TRAF6 (Tumor Necrosis Factor receptor associated factor 6) is an E3 ubiquitin ligase that is essential for signaling downstream of the interleukin-1 (IL-1) receptor/Toll-like receptor (IL-1R/TLR) superfamily and of specific members of the TNF receptor superfamily (CD40 and RANK)\(^{18,19}\). Specifically for TLR signaling, TRAF6 participates in both the MyD88- and TRIF-dependent pathways and therefore plays a crucial role in signaling by all TLRs\(^{20}\). In the present study we addressed the cell specific function of TRAF6 in atherosclerosis using genetic mouse models. Our results revealed opposing functions of TRAF6-mediated TLR signaling in endothelial cells and macrophages. Endothelial cell specific TRAF6 deficiency reduced the severity of atherosclerosis in female ApoE\(^{-/-}\) mice by inhibiting TLR-induced NF-κB-mediated expression of proinflammatory mediators in the vascular endothelium. In contrast, both male and female ApoE\(^{-/-}\) mice lacking TRAF6 specifically in myeloid cells surprisingly showed more severe atherosclerosis. We found that TRAF6-deficient macrophages showed reduced IL-10 expression, increased ER stress and increased apoptosis when stimulated with oxidized LDL, and also displayed a reduced capacity to scavenge apoptotic bodies \textit{in vitro}. These results suggest that TLR-mediated TRAF6 signaling in macrophages is atheroprotective by regulating both the anti-inflammatory response and the survival and efferocytosis capacity of macrophages \textit{in vivo}.

\textbf{Methods}

Detailed Methods can be found in the online-only \textbf{Data Supplement}.

\textbf{Mice and diet}

Mice with conditional loxP-flanked Traf6 (\textit{Traf6}\(^{\text{FL}}\)) alleles were generated by gene targeting in C57Bl/6 (Bruce4) ES cells and were crossed with Tie2CreER\(^{\text{T2}}\)\(^{21}\) or LysMCre\(^{22}\) to generate
endothelial- and myeloid-specific TRAF6 knockouts, respectively. To study the role of TRAF6 in atherosclerosis, these mice were then crossed with $ApoE^{-/-}$ mice. For induction of Cre-mediated DNA recombination, mice carrying the Tie2CreER$^{T2}$ transgene and their Cre-negative littermates were fed a tamoxifen-containing diet for 6 weeks starting at the age of 6-7 weeks. For atherosclerosis development all mice were placed on HCD for 10 weeks before sacrifice. All animal procedures were conducted in accordance with European, national, and institutional guidelines, and protocols and were approved by local governmental authorities.

**Additional Methods**

The expanded Methods section in the online-only Data Supplement contains information on the mice and the diet used; lipid analysis; immunostainings; histology; assessments of lesion size; oxidation and labeling of LDL; isolation and culture of cells; adhesion assay; apoptosis assay; efferocytosis assay; foam cell formation assay; immunoblot analysis; southern blotting and qRT-PCR analysis.

**Statistical Analysis**

Continuous variables were summarised by mean±SEM. Regarding mean values, pairwise comparisons of groups were either evaluated by the unpaired $t$-test or non-parametric Mann-Whitney test contingent on the presence of heavy tails or outliers / skewness. Moreover, multiple pairwise comparisons of groups over time were evaluated by repeated measures 2-way ANOVA with Bonferroni post-hoc tests (corrected p values are given for comparison between genotypes at each time point). The usual family wise type-I error of 5% was adopted to assess statistical significance. All analyses were done with Prism (GraphPad Software Inc., San Diego, CA).
Results

TRAF6 ablation in endothelial cells reduced atherosclerotic plaque development in female ApoE<sup>−/−</sup> mice.

To study in vivo the role of TRAF6 in the vascular endothelium we used mice with inducible endothelial cell-restricted TRAF6 deficiency (TRAF6<sup>ECiKO</sup>), generated by crossing mice with loxP-flanked TRAF6 alleles (Supplemental Figure 1A) to Tie2-CreER<sup>T2</sup> mice<sup>21</sup>. TRAF6<sup>ECiKO</sup> mice were further crossed to ApoE<sup>−/−</sup> mice, a widely used mouse model of atherosclerosis. Cre-mediated excision of the floxed TRAF6 alleles in endothelial cells (Supplemental Figures 1B-C) was achieved by feeding groups of TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> and their TRAF6<sup>FL/FL/ApoE<sup>−/−</sup></sup> littermates (hereafter referred to as ApoE<sup>−/−</sup> for simplicity) with tamoxifen-containing chow diet for 6 weeks as previously described<sup>23</sup>. The mice were subsequently placed on high fat diet (HFD) for a period of 10 weeks. TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> and ApoE<sup>−/−</sup> mice showed similar body weight and serum levels of triglycerides and cholesterol after HFD treatment (Figures 1A-C), indicating that endothelial-specific deletion of TRAF6 did not affect basic metabolic features.

Histological analysis of heart sections at the level of the aortic sinus showed significantly decreased atherosclerotic lesion development in female TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice compared to their female ApoE<sup>−/−</sup> littermates (Figures 1D-E, and Supplemental Figures 2A-B). In contrast, male TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice showed similar levels of atherosclerosis compared to their male ApoE<sup>−/−</sup> littermates (Figures 1D-E and Supplemental Figures 2A-B). Importantly, control experiments in ApoE<sup>−/−</sup>/Tie2CreER<sup>T2</sup> mice that did not contain TRAF6<sup>FL</sup> alleles showed that neither tamoxifen treatment nor Tie2-CreER<sup>T2</sup> expression had a measurable effect in the development of atherosclerosis in this model (Supplemental Figures 3A-B). Thus, endothelial
specific TRAF6-deficiency could reduce the severity of atherosclerosis in female but not in male mice.

Reduced chemokine and adhesion molecule expression and macrophage recruitment in plaques from female TRAF6ECiKO/ApoE−/− mice

To dissect the mechanisms by which endothelial TRAF6 deficiency reduced atherosclerosis severity we focused on the analysis of female TRAF6ECiKO/ApoE−/− mice. We hypothesized that endothelial TRAF6 deficiency reduced atherosclerosis severity by inhibiting the expression of chemokines and adhesion molecules in the endothelium resulting in impaired recruitment of macrophages to the developing plaques. Indeed, immunostaining with MOMA-2 antibodies revealed considerably decreased macrophage content in plaques from female TRAF6ECiKO/ApoE−/− mice compared to their female ApoE−/− littermates (Figures 2A-B). Gene expression analysis revealed reduced expression of a number of chemokines including IP-10, MIP-1a/1b/2a, Fraktalkine and Gro-KC in aortas from female TRAF6ECiKO/ApoE−/− mice compared to their ApoE−/− littermates. Moreover, IL-6 and VCAM-1 were also expressed at considerably lower levels in aortas from TRAF6ECiKO/ApoE−/− mice (Figure 2C-E). Interestingly, increased expression of TGFβ was observed in the aortas of TRAF6ECiKO/ApoE−/− mice, arguing for enhanced anti-inflammatory responses in these mice. These results suggest that ablation of TRAF6 in endothelial cells ameliorated atherosclerotic plaque development by inhibiting the expression of VCAM-1 and of several chemokines in the vascular endothelium, resulting in impaired macrophage recruitment into the developing plaques.

TRAF6 deficiency in endothelial cells inhibited oxidized LDL-induced NF-κB activation, inflammatory gene expression and monocyte/macrophage adhesion.

We previously demonstrated that NF-κB inhibition in endothelial cells protects ApoE−/− mice.
from atherosclerosis by inhibiting the expression of adhesion molecules and chemokines in the vascular wall resulting in impaired macrophage recruitment into the plaques\textsuperscript{23}. Modified lipids were shown to bind TLRs\textsuperscript{24} and activate NF-κB signaling in endothelial cells\textsuperscript{25}. We therefore reasoned that TRAF6 deficiency reduced atherosclerosis by inhibiting modified lipid-induced NF-κB activation and the expression of pro-inflammatory mediators in endothelial cells. To address whether TRAF6 deficiency inhibits oxLDL-induced inflammatory responses we analyzed wild type and TRAF6-deficient primary mouse lung endothelial cells (MLEC). Immunoblotting of protein extracts with antibodies recognizing the phosphorylated forms of IκBα and JNK showed that TRAF6-deficient MLEC showed strongly reduced NF-κB and JNK activation in response to oxLDL stimulation compared to MLEC expressing TRAF6 (Figure 3A). Moreover, TRAF6-deficiency strongly inhibited oxLDL-induced expression of VCAM-1, ICAM-1, E-selectin, MCP1 and MCP3 in MLECs (Figures 3B-F). To directly address the capacity of oxLDL-stimulated TRAF6-deficient endothelial cells to attract monocytes, we used a static-adhesion assay measuring the ability of wild type monocytes to adhere on wild type or TRAF6-deficient endothelial cells. This experiment revealed that monocyte adhesion on oxLDL-stimulated \textit{Traf6\textsuperscript{−/−}/ApoE\textsuperscript{−/−}} MLEC was considerably reduced compared to \textit{ApoE\textsuperscript{−/−}} MLEC (Figure 3G). Therefore, TRAF6 is essential for NF-κB activation and the expression of chemoattractant chemokines and adhesion molecules in endothelial cells in response to oxLDL-induced TLR stimulation.

**Macrophage TRAF6 deficiency exacerbates atherosclerosis development**

Our results described above revealed a pro-atherogenic role of TRAF6-dependent signaling in endothelial cells, acting downstream of TLRs to induce an NF-κB-dependent proinflammatory gene expression program that facilitates the recruitment of monocytes into the developing
plaques. We then asked whether TRAF6 also acts in myeloid cells to promote atherosclerosis. To address the role of TRAF6 in myeloid cells in the pathogenesis of atherosclerosis, we crossed $Traf6^{FL/FL}/ApoE^{-/-}$ mice with LysM-Cre mice. These TRAF6$^{MYKO}/ApoE^{-/-}$ mice showed efficient deletion of TRAF6 in peritoneal macrophages, as revealed by southern blot and immunoblot analysis (Supplemental Figures 1D-E). To study the development of atherosclerosis, groups of TRAF6$^{MYKO}/ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates were fed a HFD for 10 weeks starting at 6 weeks of age. Both groups of mice showed similar body weight and serum levels of triglycerides and total cholesterol upon feeding with HFD (Figures 4A-C) demonstrating that myeloid TRAF6 deficiency did not affect basic metabolic features. After 10 weeks of HFD feeding we assessed atherosclerosis development by histological analysis of heart sections at the level of the aortic sinus. Quantification of atherosclerotic plaque size in the aortic sinus revealed that both male and female TRAF6$^{MYKO}/ApoE^{-/-}$ mice developed more severe atherosclerosis compared to their $ApoE^{-/-}$ littermate controls (Figures 4D-E, and Supplemental Figures 4A-B). This result was surprising and contrary to our expectation that myeloid TRAF6 deficiency would have an atheroprotective effect. To address whether TRAF6 deficiency increased macrophage homing into the plaques we stained aortic sinus sections from TRAF6$^{MYKO}/ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermate controls with anti-MOMA-2 antibodies. Plaques in both groups of mice showed similar macrophage content, suggesting that TRAF6-deficiency did not alter the capacity of macrophages to home into the developing lesions (Figures 4F-G).

**Reduced expression of IL-10 in aortas from TRAF6$^{MYKO}/ApoE^{-/-}$ mice**

To obtain clues about the potential mechanisms by which TRAF6 knockout in myeloid cells exacerbates atherosclerosis we measured the expression of chemokines, cytokines and adhesion molecules in RNA isolated from aortic branches of $ApoE^{-/-}$ or TRAF6$^{MYKO}/ApoE^{-/-}$
littermates fed with a HFD for 10 weeks. This analysis did not reveal statistically significant
differences in the expression of pro-atherogenic cytokines, chemokines and adhesion
molecules between the two groups in either male (Figures 5A-C) or female (Supplemental
Figures 5A-C) mice. Nevertheless, we observed a statistically significant decrease in the
expression levels of the anti-inflammatory cytokine IL-10 in aortas from TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{-/-}
mice compared to their ApoE\textsuperscript{-/-} littermates in both male (Figure 5A) and female
(Supplemental Figure 5A) mice. To assess whether the observed decrease in IL-10
production in the aortas was caused by an impairment of IL-10 expression in TRAF6-
deficient macrophages, we stimulated TRAF6-deficient or wild type bone marrow derived
macrophages (BMDM) with oxidized LDL and measured activation of NF-κB and JNK and
the expression of IL-10, TNF and IL-6. As shown in Figure 5D, IκBα phosphorylation and
degradation was delayed and reduced and JNK phosphorylation was strongly inhibited in
TRAF6-deficient macrophages. In addition, RNA expression analysis revealed that Traf6\textsuperscript{-/-}
macrophages expressed considerably reduced levels of IL-10 compared to control cells after
stimulation with oxLDL (Fig. 5E). TRAF6-deficient macrophages also expressed mildly
reduced levels of IL-6 and TNF (Figures 5F-G) as well as CD11a (Supplemental Figure
6B) compared to wild type cells. Thus, TRAF6 deficiency in macrophages resulted in
decreased IL-10 expression in atherosclerotic plaques \textit{in vivo} and in cultured macrophages.
Since IL-10 has been shown to negatively regulate atherosclerotic plaque development\textsuperscript{26},
reduced IL-10 expression is likely to contribute to the development of more severe
atherosclerosis in TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{-/-} mice.
TRAF6-deficient macrophages show increased sensitivity to ER stress and impaired efferocytosis

IKK2 deficiency in macrophages resulted in exacerbated atherosclerotic plaque development associated with reduced expression of IL-10 \(^{27}\), similarly to our findings with TRAF6\(^{MYKO}/ApoE^{-/-}\) presented here. In addition, mice with myeloid IKK2-deficiency showed increased apoptosis in atherosclerotic plaques \(^{27}\). We therefore assessed whether TRAF6 deficiency in macrophages also resulted in increased cell death in atherosclerotic plaques by measuring the necrotic core area in lesions from TRAF6\(^{MYKO}/ApoE^{-/-}\) mice and their \(ApoE^{-/-}\) littermates. The atherosclerotic lesions in aortas from TRAF6\(^{MYKO}/ApoE^{-/-}\) mice contained increased necrotic core area (Figures 6A-B and Supplemental Figures 4C-D) and increased numbers of cells showing activation of caspase 3 (Figures 6C-D) compared to lesions from \(ApoE^{-/-}\) mice, suggesting that increased sensitivity of TRAF6-deficient macrophages to apoptosis could contribute to the development of more severe atherosclerosis in TRAF6\(^{MYKO}/ApoE^{-/-}\) mice.

A recent study showing that TLR signaling suppresses endoplasmic reticulum (ER) stress-induced CHOP expression and death in macrophages \(^{28}\) prompted us to test whether TRAF6 deficiency affects the expression of CHOP in atherosclerotic plaques. Indeed, we found increased CHOP expression in lesion areas from both male (Figure 6E) and female (Supplemental Figure 5D) TRAF6\(^{MYKO}/ApoE^{-/-}\) mice, suggesting that TRAF6 deficiency results in elevated CHOP levels in macrophages. Since analysis of total aorta tissue cannot address specifically the role of TRAF6 in controlling CHOP expression in macrophages, we used BMDM cultures to directly address whether loss of TRAF6 results in increased CHOP levels. \(Traf6^{FL/FL}\) or \(Traf6^{-/-}\) bone marrow macrophages were pre-stimulated with 2 ng/ml LPS for 20
hours followed by stimulation with 3μg/ml tunicamycin to induce ER stress. Immunoblot analysis of protein extracts prepared 3, 6 or 9 hours after tunicamycin stimulation showed that LPS pre-treatment indeed prevented CHOP expression in wild type macrophages (Figure 6F), as previously reported28. In contrast, LPS pre-treatment failed to suppress CHOP expression in macrophages lacking TRAF6, demonstrating that TRAF6 is an essential mediator of TLR4-induced suppression of CHOP expression. Furthermore, TRAF6-deficient BMDMs expressed increased amounts of CHOP mRNA compared to wild type cells in response to oxLDL stimulation (Figure 6G). Increased ER-stress as well as defective IL-10 signaling may lead to increased apoptosis of macrophages both in vitro and in vivo. Indeed, oxLDL stimulation induced more apoptosis in peritoneal macrophages from TRAF6MYKO/ApoE−/− mice compared to ApoE−/− cells (Figure 6H). The differential responses of TRAF6-deficient macrophages to oxLDL stimulation were not caused by an impairment of oxLDL uptake, as these cells retained a normal capacity to take up oxLDL compared to wild type macrophages (Figure 7A).

Clearance of apoptotic macrophages is an important mechanism limiting plaque growth and atherosclerosis progression29. To assess whether TRAF6 deficiency compromised macrophage efferocytosis, we measured the capacity of TRAF6-deficient macrophages to clear apoptotic cells. Indeed, peritoneal macrophages from TRAF6MYKO/ApoE−/− mice exhibited reduced efficiency to clear apoptotic Jurkat T cells compared to peritoneal macrophages from ApoE−/− mice (Figure 7B). Scavenger receptors have been previously implicated in the recognition and clearance of apoptotic cells30, 31. Consistent with their impaired efferocytosis capacity, TRAF6-deficient naïve peritoneal macrophages expressed reduced amounts of SR-AI/II (Figures 7D-E) and CD36 (Figures 7F-G). This reduced cell surface expression of scavenger receptors did not affect the formation of foam cells by Traf6−/−
ApoE−/− macrophages in vitro (Figure 7H-I). IL-10 within the atherosclerotic lesions is also produced from macrophages during efferocytosis32. Considering that we found reduced IL-10 expression in aortic lesions from TRAF6MYKO/ApoE−/− mice, we tested whether TRAF6-deficient macrophages exhibit reduced IL-10 expression upon exposure to apoptotic cells. Indeed, we found that TRAF6-deficient macrophages failed to upregulate IL-10 expression when stimulated by apoptotic Jurkat T cells (Figure 7C). Taken together, these results show that TRAF6 deficiency sensitized macrophages to oxLDL-induced apoptosis and reduced their efferocytosis and anti-inflammatory properties resulting in exacerbated atherosclerosis.

Discussion

The development and growth of an atherosclerotic plaque is the result of a chronic non-resolving inflammatory response involving the activation of the vascular endothelium at the early stages, a constant influx and entrapment of monocytes and impaired clearance of dying macrophages within the plaque. Therefore, the mechanisms regulating the recruitment of monocytes and the death and clearance of lipid-laden macrophages in atheromatous plaques are critical for the pathogenesis of atherosclerosis. Our results presented here show that TRAF6-dependent TLR signaling exerts opposing functions in endothelial cells and macrophages in atherosclerosis. TRAF6-deficiency in endothelial cells inhibited the expression of pro-inflammatory mediators and of the adhesion molecule VCAM-1 resulting in reduced macrophage influx and smaller atherosclerotic plaques in female mice. Moreover, TRAF6-deficient primary endothelial cells showed impaired expression of pro-inflammatory cytokines and chemokines and adhesion molecules after stimulation with oxLDL. The expression pattern of proinflammatory mediators in the aortas of TRAF6ECiKO/ApoE−/− mice resembles the transcriptional profile previously reported in MyD88−/−/ApoE−/− mice12, suggesting that endothelial-specific inhibition of TRAF6-
dependent TLR signaling protects mice from the development of atherosclerosis by preventing the upregulation of cytokines, chemokines and adhesion molecules coordinating macrophage influx into the plaque. Consistent with these results, we previously showed that NF-κB inhibition specifically in vascular endothelial cells protected ApoE/− mice from atherosclerosis by preventing the expression of proinflammatory factors and the recruitment of monocytes to the developing plaques. Together, these studies suggest that TLRs on the surface of endothelial cells sense modified lipids and activate NF-κB signaling in a TRAF6-dependent manner, inducing proinflammatory mediator expression and the recruitment of monocytes into the lesion facilitating plaque growth. Curiously, the atheroprotective effect of endothelial TRAF6 deficiency was observed only in female mice, which are known to develop more severe atherosclerosis than males. Endothelial TRAF6 deficiency reduced atherosclerosis in female mice to levels that are typically seen in males, suggesting that TRAF6 ablation could prevent plaque growth above a certain threshold that was not reached in male mice, hence the lack of a measurable effect in this group. This is in contrast to the effect of endothelial specific NF-κB inhibition that very strongly reduced atherosclerosis in both males and females. The stronger effect of NF-κB inhibition is not unexpected, as TLR signaling is only one of the upstream pathways that can induce NF-κB in the endothelium during atherosclerotic plaque development. Monocytes and stromal cells also sense modified lipids and express cytokines that can act on endothelial cells to induce proinflammatory gene expression in a TLR-independent fashion. Therefore, inhibition of TLR signaling is expected to affect only one of the pathways that coordinate proinflammatory gene expression in the vascular endothelium, while NF-κB inhibition is expected to block nearly every upstream pathway activating the expression of proinflammatory mediators. Thus, TLR-mediated NF-κB activation in endothelial cells seems to
have an important role in regulating the growth of atherosclerotic plaques, but other TLR-independent pathways acting on the vascular endothelium also contribute to the formation of atherosclerotic lesions.

While TRAF6 deficiency in endothelial cells protected mice from atherosclerosis, TRAF6 deficiency in macrophages surprisingly exacerbated atherosclerosis in ApoE^{-/-} mice. This result was unexpected and provocative, particularly considering the atheroprotective effect observed in mice with complete knockout of MyD88, TLR4 or TLR2, which indicated that TLR signaling plays an overall pathogenic role in atherosclerosis. Our studies suggest that TRAF6 deficiency in macrophages exacerbated atherosclerotic plaque development via different mechanisms. On the one hand TRAF6-deficient macrophages showed reduced IL-10 expression upon stimulation of oxLDL. This is most likely due to inhibition of TRAF6-dependent TLR signaling, as MyD88-deficient macrophages also showed impaired IL-10 expression upon oxLDL stimulation (Supplemental Figure 7). Given the anti-inflammatory function of IL-10 and the increased atherosclerosis observed in IL-10 deficient mice^{26}, the reduced levels of IL-10 are likely to contribute to the development of more severe atherosclerosis in TRAF6^{MYKO/ApoE^{-/-}} mice. On the other hand, TRAF6^{MYKO/ApoE^{-/-}} mice showed increased necrotic areas in atherosclerotic plaques, suggesting that TRAF6 deficiency sensitized macrophages to cell death. Plaques from TRAF6^{MYKO/ApoE^{-/-}} mice also showed elevated CHOP expression indicating that increased ER stress might be implicated in sensitizing TRAF6-deficient macrophages to death.

Consistent with these in vivo findings, cultured TRAF6-deficient macrophages also showed increased CHOP expression and apoptosis upon stimulation with oxLDL. Previous studies showed that LPS pre-stimulation protects macrophages from ER stress by suppressing the ATF4-CHOP branch of the unfolded protein response in a TRIF-dependent manner^{28}. LPS pre-
stimulation failed to suppress CHOP expression in tunicamycin treated TRAF6-deficient macrophages demonstrating that TRAF6 is essential for the TLR-mediated protection of macrophages from ER stress. Necrotic death of lipid-laden macrophages within the plaque due to increased expression of Chop is considered an important factor contributing to plaque growth. Moreover, ApoE−/− mice lacking CHOP developed smaller and less advanced atherosclerotic lesions, further supporting a critical role of the UPR in atherosclerosis. Thus, increased ER stress and apoptosis of TRAF6-deficient macrophages within the developing plaques is expected to have a pro-inflammatory effect and increase the severity of atherosclerosis.

Reduced efferocytosis is considered to be a major contributor to the development to the necrotic core, leading to plaque vulnerability. We found that macrophages lacking TRAF6 showed reduced capacity to scavenge apoptotic debris and expressed reduced levels of IL-10 when exposed to apoptotic cells. Since IL-10 expression is also linked to efferocytosis and the anti-inflammatory response of efferocytes within the plaques, impaired IL-10 expression is likely to be at least in part responsible for the reduced clearance of apoptotic macrophages in TRAF6MYKO/ApoE−/− mice. In addition, we showed that TRAF6-deficient macrophages expressed reduced levels of SR-AI/II and CD36, scavenger receptors that have been implicated in the recognition of apoptotic bodies by macrophages and efferocytosis. Thus, impaired scavenger receptor expression together with reduced IL-10 levels inhibited the efferocytosis capacity of TRAF6-deficient macrophages contributing to the formation of a larger necrotic core and more severe atherosclerosis in TRAF6MYKO/ApoE−/− mice.

In contrast to our findings reported here, Zirlik and colleagues reported previously that LDL receptor knockout mice reconstituted with TRAF6-deficient fetal liver cells developed similar levels of atherosclerosis as mice reconstituted with wild type fetal liver. This apparent
discrepancy is most likely due to the inherent differences in the experimental systems used. Zirlik and colleagues showed that Ldlr<sup>-/-</sup> mice reconstituted with TRAF6-deficient fetal liver weighed about 30% less than Ldlr<sup>-/-</sup> mice reconstituted with wild type fetal liver and showed reduced lipid levels in the blood. We did not observe any effect of myeloid or endothelial TRAF6 deficiency in weight gain or lipid levels, suggesting the effect observed by Zirlik and colleagues is likely caused by the lack of TRAF6 in hematopoietic derived cells other than macrophages. Mice reconstituted with TRAF6-deficient fetal liver were previously shown to develop a progressively lethal inflammatory disease starting as early as 4 weeks post-reconstitution<sup>35,36</sup>. Moreover, mice with T cell-specific TRAF6 deficiency developed a multiorgan inflammatory disease by 10-12 weeks after birth due to a role of TRAF6 in the maintenance of T cell homeostasis<sup>36</sup>. It is likely that the reduced weight of TRAF6-deficient fetal liver chimeras reported by Zirlik and colleagues is also due to the development of a similar multiorgan inflammation, which could affect the development of atherosclerosis and explain their different findings compared to our study.

TRAF6 is a critical adapter molecule not only for TLRs but also for a number of cytokine receptors including CD40<sup>19</sup>. The role of the CD40/TRAF6 axis in atherosclerosis has been previously studied in mouse models expressing different CD40 mutants that have differential capacity to activate distinct downstream signaling cascades. Lutgens et al reported that <i>ApoE</i><sup>-/-</sup> mice expressing a CD40 mutant unable to induce TRAF6-mediated signaling were protected from atherosclerosis (surprisingly these mice were even more protected than <i>ApoE</i><sup>-/-</sup>/<i>CD40</i><sup>-/-</sup> mice), suggesting that CD40 – TRAF6 signaling is an important pathogenic factor in atherosclerosis<sup>37</sup>. These authors suggested that inhibition of the CD40-TRAF6 axis in MHC+ myeloid cells is responsible for the observed protective effect in their mice. Our findings that
mice with myeloid-specific knockout of TRAF6 develop more severe atherosclerosis
demonstrate that other upstream receptors, most likely TLRs, play a prominent role in inducing
TRAF6-dependent signals that overshadow the potential role of the CD40 – TRAF6 axis in
regulating myeloid cell responses in atherosclerosis.

Taken together, our results unravel a previously unanticipated complexity on the role of
TRAF6 as a downstream mediator of TLR signaling pathways in the development of
atherosclerosis. The TLR/TRAF6/IKK/NF-κB pathway plays a predominantly pathogenic role in
the development of atherosclerosis in female mice by acting in endothelial cells, where it senses
modified lipids and induces the expression of proinflammatory mediators that sustain the
constant adhesion and recruitment of macrophages promoting plaque growth. On the other hand,
TRAF6 deficiency in myeloid cells exacerbates the development of atherosclerosis in both males
and females by inhibiting the expression of IL-10, sensitizing macrophages to ER-stress-induced
death and reducing their efferocytosis capacity. Thus, TLR-induced signals converge on TRAF6
to regulate the pathogenesis of atherosclerosis by controlling cell-specific cellular responses that
exert diverse and opposing effects in plaque development.

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References:


Figure Legends:

Figure 1. Endothelial-specific TRAF6 ablation reduces HFD-induced atherosclerosis in ApoE<sup>−/−</sup> mice. (A) Body weight and (B) serum triglyceride levels of ApoE<sup>−/−</sup> and TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice after 10 weeks of HFD. (C) Serum cholesterol levels in ApoE<sup>−/−</sup> and TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice. BT: Before tamoxifen; 0w: After tamoxifen and before HFD; 10w: After 10 weeks on HFD. ApoE<sup>−/−</sup> males, n=9; TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> males, n=10; ApoE<sup>−/−</sup> females, n=11; TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> females, n=12. (D) Representative aortal cross-sections at the height of the aortic sinus from ApoE<sup>−/−</sup> and TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice. Scale bars=0.5mm. (E) Graph showing quantification of atherosclerotic lesion size at the aortic sinus of ApoE<sup>−/−</sup> and TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice. Results represent pooled data from two independent groups of mice (Mann-Whitney test).

Figure 2. Reduced macrophage accumulation and inflammation in the aortic lesions of TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice. (A) Representative pictures from immunostainings of atherosclerotic lesions of female ApoE<sup>−/−</sup> or TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice with MOMA-2 antibodies. Scale bars=0.1mm. (B) Graph showing quantification of macrophage content in the lesions of female ApoE<sup>−/−</sup> or TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice (unpaired t-test). (C-E) qRT-PCR analysis of adhesion molecules (C), cytokines (D) and chemokines (E) in the aortas of female TRAF6<sup>ECiKO/ApoE<sup>−/−</sup></sup> mice compared to female ApoE<sup>−/−</sup> mice (Mann-Whitney test).

Figure 3. TRAF6 deficiency inhibits oxLDL-induced inflammatory responses in endothelial cells. (A) Immunoblot analysis of whole cell protein extracts from MLEC after stimulation with 100 μg/ml of oxLDL with the indicated antibodies. One representative of three independent
experiments shown. (B-F) qRT-PCR analysis shows reduced expression of VCAM-1 (B), ICAM-1 (C), E-Selectin (D), MCP-1 (E) and MCP-3 (F) in Traf6<sup>-/-</sup>/ApoE<sup>-/-</sup> MLEC after stimulation with oxLDL. Pooled data from three independent experiments shown (n=7 per genotype, 2-way ANOVA with Bonferroni post-hoc tests comparing ApoE<sup>-/-</sup> and Traf6<sup>-/-</sup>/ApoE<sup>-/-</sup> MLEC at each time point). (G) Graph showing the relative adhesion of bone marrow cells on oxLDL-stimulated MLEC (n=3 per genotype, 2-way ANOVA with Bonferroni post-hoc tests). This experiment was performed twice with similar results. *p<0.05 **p<0.01 ***p<0.001.

**Figure 4.** Myeloid-specific TRAF6 deficiency exacerbates HFD-induced atherosclerosis in ApoE<sup>-/-</sup> mice. (A) Body weight and (B) serum triglyceride levels of ApoE<sup>-/-</sup> and TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice after 10 weeks of HFD. (C) Serum cholesterol levels in ApoE<sup>-/-</sup> and TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice. 0w: Before HFD; 10w: After 10 weeks on HFD. (D) Representative aortal cross-sections at the height of the aortic sinus of ApoE<sup>-/-</sup> and TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice. Scale bars=0.5 mm. (E) Graph showing quantification of atherosclerotic lesion size at the aortic sinus of ApoE<sup>-/-</sup> and TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice. Results represent pooled data from two independent groups (Mann-Whitney test). (F) Representative images from immunostainings of atherosclerotic lesions of male ApoE<sup>-/-</sup> or TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice with MOMA-2 antibodies. Scale bars=0.1 mm. (G) Graph showing quantification of macrophage content in the lesions of male ApoE<sup>-/-</sup> or TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> mice.

**Figure 5.** TRAF6 deficient myeloid cells show impaired NF-κB and JNK activation and reduced IL-10 expression upon oxLDL stimulation. (A-C) qRT-PCR analysis of cytokines (A), chemokines (B) and adhesion molecules (C) in the aortas of male TRAF6<sup>MYKO</sup>/ApoE<sup>-/-</sup> and...
ApoE−/− mice (Mann-Whitney test). (D) Immunoblot analysis of protein extracts from bone
marrow macrophages after stimulation with 50 μg/ml of oxLDL with the indicated antibodies.
One representative of three independent experiments shown. (E-G) qRT-PCR analysis of IL-10
(E), IL-6 (F) and TNF (G) expression in Traf6−/− and control Traf6FL/FL macrophages after
stimulation with 50 μg/ml of oxLDL. One representative of three independent experiments
shown (n=3 per genotype, 2-way ANOVA with Bonferroni post-hoc tests comparing Traf6−/− and
control Traf6FL/FL macrophages at each time point). Statistical significance is indicated by
asterisks placed above the specific time points. *p<0.05 **p<0.01 ***p<0.001.

**Figure 6.** TRAF6 deficiency increases ER stress-induced macrophage death *in vivo* and *in vitro.*
(A) Representative histological images from Toluidine stained aortic lesions of male ApoE−/− or
TRAF6MYKO/ApoE−/− mice. Scale bars=0.1 mm. (B) Graph showing quantification of the necrotic
core area in lesions from ApoE−/− and TRAF6MYKO/ApoE−/− mice (pooled data from two
independent groups of mice, Mann-Whitney test). (C) Representative images from aortic lesions
from male ApoE−/− and TRAF6MYKO/ApoE−/− mice immunostained with antibodies recognising
activated caspase-3. Scale bars=0.1 mm. (D) Graph showing quantification of cells with active
caspase-3 in male ApoE−/− and TRAF6MYKO/ApoE−/− mice (unpaired t-test). (E) Graph showing the
relative expression levels of CHOP mRNA in aortas of male ApoE−/− or TRAF6MYKO/ApoE−/−
mice after 10 weeks of HFD (Mann-Whitney test). (F) Bone marrow macrophages were pre-
incubated with LPS (2 ng/ml) for 20 hours and then stimulated with tunicamycin (3 μg/ml) for 3,
6 or 9 hours. Whole cell protein extracts were analysed by immunoblotting using the indicated
antibodies. One representative of two independent experiments is shown. (G) Graph showing the
relative expression levels of CHOP mRNA in Traf6−/− and Traf6FL/FL macrophages after
stimulation with oxLDL (50 μg/ml) (n=3 per genotype, 2-way ANOVA with Bonferroni post-hoc tests comparing Traf6<sup>−/−</sup> and control Traf6<sup>FL/FL</sup> macrophages at each time point). Statistical significance is indicated by asterisks placed above the specific time points. One representative of three independent experiments is shown. ***p<0.001. (H) Peritoneal macrophages were isolated form ApoE<sup>−/−</sup> or TRAF6<sup>MYKO</sup>/ApoE<sup>−/−</sup> mice and stimulated with oxLDL (100 μg/ml) for 48 hours. Apoptosis was quantified using FACS analysis of Annexin-V. One representative result of three independent experiments shown. (n=5 per genotype, 2-way ANOVA with Bonferroni post-hoc tests).

**Figure 7.** TRAF6 deficiency inhibits scavenger receptor expression and efferocytosis in macrophages. (A) Graph showing quantification of the uptake of Dil-labeled oxLDL by ApoE<sup>−/−</sup> or Traf6<sup>−/−</sup>/ApoE<sup>−/−</sup> peritoneal macrophages. One representative of two independent experiments is shown (n=4 per genotype, 2-way ANOVA with Bonferroni post-hoc tests). (B) Graph showing quantification of efferocytosis of apoptotic Jurkat T cells by ApoE<sup>−/−</sup> or Traf6<sup>−/−</sup>/ApoE<sup>−/−</sup> peritoneal macrophages. One representative of two independent experiments shown (n=4 per genotype, unpaired t-test). (C) qRT-PCR analysis of IL-10 expression in bone marrow macrophages upon incubation with apoptotic Jurkat T cells. One representative of two independent experiments shown (n=3 per genotype, 2-way ANOVA with Bonferroni post-hoc tests comparing Traf6<sup>−/−</sup> and control Traf6<sup>FL/FL</sup> macrophages at each time point). Statistical significance is indicated by asterisks placed above the specific time points. **p<0.01, ***p<0.001. (D-E) Representative overlay histogram (D) and quantification (E) of SR-AI/II expression on the cell-surface of naïve peritoneal macrophages isolated form ApoE<sup>−/−</sup> or TRAF6<sup>MYKO</sup>/ApoE<sup>−/−</sup> mice (n=6 per genotype, unpaired t-test). (F-G) Representative overlay histogram (F) and quantification (G) of CD36
expression on the cell-surface of naïve peritoneal macrophages isolated form \( ApoE^{-/-} \) or TRAF6\(^{MYKO}/ApoE^{-/-} \) mice (n=6 per genotype, unpaired t-test). (H) Representative pictures (BODIPY 493/503 and DAPI double staining) of peritoneal macrophages isolated form \( ApoE^{-/-} \) or TRAF6\(^{MYKO}/ApoE^{-/-} \) mice after stimulation for 48 hours with 50 mg/ml of oxidized-LDL or normal medium, and quantification (I) of foam cells after oxidized-LDL stimulation (n=6 per genotype, unpaired t-test).
Conditional Targeting of TRAF6 Reveals Opposing Functions of TLR Signaling in Endothelial and Myeloid Cells in a Mouse Model of Atherosclerosis

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Mice and diet

Mice with conditional loxP-flanked Traf6 alleles were generated by gene targeting in Bruce 4 ES cells derived from C57Bl/6 mice (Supplemental Figure 1) and were crossed with the Tie2CreERT2 or the LysMCre2 transgenic lines to generate mice with endothelial and myeloid cell-specific TRAF6 deficiency, respectively. In order to allow studying the effect of TRAF6 deficiency on atherosclerosis, these mice were then crossed with ApoE−/− mice, which constitute a widely accepted mouse model of the disease. Tie2CreERT2, LysMCre and ApoE−/− mice were backcrossed at least 10 times in the C57Bl/6 background. For induction of Cre activity, mice carrying the Tie2CreERT2 transgene and their Cre-negative littermates were fed a tamoxifen-containing diet for 6 weeks starting at the age of 6-7 weeks as previously described.

Subsequently, the mice were placed on a western-type diet (Harland Tekland, TD88137 high cholesterol diet hereafter mentioned as HCD) for 10 weeks, to accelerate the development of atherosclerotic lesions, as previously described.

Myeloid-specific TRAF6 knockout mice were placed on HCD for 10 weeks starting at the age of 6-7 weeks. All in vivo experiments were performed twice by analyzing two independent groups of littermates with 7-15 mice/ sex/ genotype. In all experiments hearts and sera were collected for further analysis. Aortic arches were collected and were used for gene expression analysis. For preparation of bone marrow-derived macrophages TRAF6-floxed mice were crossed to mice carrying the Mx1-Cre transgene and cells were isolated after poly(I:C)-mediated induction of recombination. All animal procedures were conducted in accordance with European,
national, and institutional guidelines, and protocols and were approved by local governmental authorities.

**Lipid analysis**

Serum cholesterol levels were measured after overnight fasting using the PAD-CHOL reagent (Roche) according to the manufacturer’s instructions.

**Triglycerides analysis**

Serum was collected from whole blood at the day of sacrifice and stored at -80°C. Quantitation of triglycerides was performed in sera samples diluted 1:2 in PBS with the Triglyceride Quantification kit (Abcam, ab65336) according to the manufacturer’s instructions.

**Immunostainings**

Frozen sections of the aortic root were fixed in ice-cold acetone for 10 minutes, dried under a ventilator, and washed with PBS. Sections were blocked in 4% FCS with Avidin D solution (Avidin/Biotin Blocking Kit; Vector Laboratories) for 30 min. Primary antibodies were anti- mouse macrophages/ monocytes (MCA519GT, Serotec) and anti- active caspase 3 (AF835, R&D Systems). Biotinylated secondary antibodies, ABC Kit Vectastain Elite, and DAB substrate (PerkinElmer, Vector, and Dako) were used. After counterstaining with haematoxylin sections were mounted with Entellon (MERCK) mounting medium.

**Histology of Plaques and Lesion Size**

Consecutive 7 μm sections of the heart in the atrioventricular valve region were collected and stained with toluidine blue, as described previously. For morphometric analysis lesion size was measured on four consecutive sections in 42 μM intervals.
using Adobe Photoshop. Each dot represents the average plaque area (four measurements per plaque) of one individual mouse. Whenever it was not possible to obtain four measurements the result was neglected though different sections of the hearts were used for further staining.

**Oxidation and labeling of LDL**

CuSO₄ oxidation and Dil-labelling of human LDL (AppliChem) was performed at 37°C according to standard protocols and as previously described.

**Isolation and culture of macrophages**

For preparation of bone marrow–derived macrophages (BMDM), bone marrow cells were subjected to red blood cell lysis and plated on 10 cm bacterial Petri dishes (Greiner) in RPMI Glutamax (Invitrogen) supplemented with 10% FCS penicillin/streptomycin and 20% L929 conditioned medium. The cells were replated in tissue-coated dishes at day 8 of culture. Experiments were performed at day 10 of culture. For poly(I:C)-mediated induction of recombination mice received two intraperitoneal injections with 250µl polyI:C /250µl PBS, with a 2 days interval and were sacrificed 4 days after the last injection. To isolate peritoneal macrophages mice were injected with 1 ml of 3% thioglycolate-Broth medium. 4 days post-injection peritoneal cells were collected after washing with ice-cold PBS and plated at the concentration of 5*10⁵ cells/well in 6-well plates in DMEM medium containing 10% FCS. 3 hours after plating non-adherent cells were removed by washing the wells with PBS. Stimulation of cells was performed on day 2 after the original plating. Further analysis was performed on the F4/80 expressing cells.

**Isolation and sorting of naïve peritoneal macrophages**
Cells were isolated by flushing the peritoneum with 8 ml ice-cold PBS and stained with antibodies against CD19-FITC (BD Pharmingen, 553785) and CD11b-PE (NatuTec, 12-0112-82). CD11b-positive cells were sorted and used for further analysis. For the analysis of the expression of scavenger receptors on the cell surface of naïve peritoneal macrophages antibodies against SR-AI/II (clone 2F8, Serotec) and CD36 (clone HM36, Novus Biological) were used.

Isolation of Mouse Lung Endothelial Cells (MLEC)

Isolation of MLEC was performed as previously described. Briefly, after perfusion with PBS lungs were dissected, passed through 75% ethanol and placed in fresh DMEM (Gibco). Using a blade, the lungs were cut into very small pieces to produce a pate consistency and subsequently incubated in 0.2% collagenase (Gibco) in DPBS + CaCl₂ (Gibco) for 1hr at 37° C. Subsequently the solution was passed through a 19G needle to break down remaining tissue and passed through a 70µM cell strainer. The cell suspension was then centrifuged at 1500 rpm for 5 min. The remaining pellet was resuspended in endothelial cell medium (DMEM low glucose: Ham’s F-12 1:1, 20% FCS, 50 µg/ml endothelial mitogen, 25 µg/ml heparin, 100U/ml penicillin/streptomycin and 2mM glutamine) and plated on a T75 flask (Corning) previously coated with coating medium (0.1% gelatin, 10 mg/ml human fibronectin, 30 mg/ml bovine collagen type I). Endothelial cells were enriched by negative (LEAF rat anti-mouse CD16/32, 1:2000 dilution, Biolegend) and positive (CD102 rat anti-mouse, 1:2000 dilution, BD Biosciences) selection using Dynabeads (sheep anti-rat IgG, Invitrogen) during passages 1-2. Endothelial cell purity was assessed by FACS after staining with the mouse endothelial cell marker WVF. At passage 5 MLEC were treated with HTN-Cre for 16 hrs in medium containing DMEM (low glucose):RPMI:PBS (in a relative ratio 1:1:2) with 100U/ml penicillin/streptomycin. The next day the medium containing HTN-Cre was removed and replaced with
normal endothelial cell medium. The cells were allowed to reach confluence and passed twice before being used for experiments. Cells were starved overnight before stimulation with oxidized LDL, unless otherwise indicated. MLEC were routinely passaged 1:3 and used up to passage 7.

**Monocyte adhesion assay**

MLEC cells were cultured until they reached confluence in 96-wells. Cells were washed and stimulated with oxLDL (100 μg/ml). 24 hours latter the medium was aspirated, cells were washed 3 times with PBS and further incubated with calcein-AM (V13181, Invitrogen) labeled macrophages (10⁵ cells/ 100 μl RPMI medium 10% FCS/ well) and for 45 minutes at 37°C. The cells were washed carefully 5 times with medium in order to remove non-adherent macrophages, further washed 3 times with PBS and lysed with 100 μl/ well of 5% NaOH. Absorbance was measured in Safire² Tecan microplate reader and the relative adhesion was after subtracting the basal auto-fluorescence of endothelial cells.

**Apoptosis assay**

Peritoneal or bone-marrow derived macrophages were stimulated with 100 μg/ml oxLDL. 48 hours later the cells were washed 2 times with annexin-V buffer, incubated with annexin V-APC (550474, BD Pharmingen) and F4/80-FITC (clone BM8, eBioscience) for 20 minutes, washed further, fixed and analysed by FACS.

**In vitro efferocytosis assay**

Jurkat cells (5*10⁶ cells/ml in RPMI medium containing 10% FCS) were labeled with calcein-AM (Molecular Probes, final concentration 5nM) at 37°C for 60 minutes according to the manufacturer's instructions. Cells were washed extensively in medium and further incubated with anti-Fas activating antibody against human Fas
(clone CH11, 100 ng/ml in RPMI medium containing 2% FCS) for 16 hours. Corporation of the calcein-AM dye as well as induction of apoptosis was evaluated by FACS (data not shown). Apoptotic Jurkat cells were washed extensively with peritoneal macrophage medium and plated on top of macrophages for 60 minutes at 37°C. At the end of the incubation the wells were washed extensively to remove excess of apoptotic Jurkat cells, stained with an F4/80-APC antibody (clone BM8, eBioscience), fixed and analysed by FACS. Efferocytosis was evaluated by the percentage of F4/80 positive cells that were also calcein-AM positive. Under the conditions used no significant basal adherence of Jurkat cells to the macrophages was observed. For RNA analysis of macrophages after stimulation with apoptotic cells the same procedure was followed without the original calcein-AM labeling of Jurkat cells.

**Foam cell formation assay**

Peritoneal macrophages were isolated after thioglycolate injection as described above. After 24 hours starvation macrophages were cultured in medium containing 2% FBS and in the presence or not of 50 μg/ml oxLDL for 48 hours. Cells were washed 3 times with PBS and fixed in 4% PFA for 20 minutes. Staining for neutral lipids was performed in the fixed cells by using the BODIPY 493/503 (Invitrogen, D-3922) according to the manufacturer’s instructions. Cells were counterstained with DAPI. Cells that contained more than 3 clear dots were considered as foam cells.

**Immunoblot analysis**

Protein lysates were prepared from macrophages and endothelial cells, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and analyzed by immunoblotting. Membranes were probed with specific antibodies against α-tubulin (Sigma-Aldrich, T6074), pIκBa (Cell Signaling, 9246), total IκBa (Santa Cruz,
sc-371), pJNK (Cell Signaling, 9251), total JNK (Cell Signaling, 9252), TRAF6 (MBL, 597) and CHOP (Santa Cruz, sc-575). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-rat secondary antibodies were used (Amersham).

**Southern Blotting**

10 µg of genomic DNA was digested with EcoRI, separated on 0.8% agarose gels and transferred to nitrocellulose. Hybridization was performed with $^{32}$P-labeled probes.

**Quantitative Real-Time PCR**

RNA was isolated from aortas and macrophage and endothelial cells using Trizol-reagent (Invitrogen) and RNeasy columns (QIAGEN). RNA (1 µg) was used for reverse transcription with SuperScript III reverse transcriptase (Invitrogen). The reaction was topped up to 200 µl with water, and 2 µl were used for quantitative real-time PCR reaction with TaqMan qPCR Kit from Eurogentec. Normalization was performed with primers for GAPDH (TaqMan).

**Statistical Analysis**

Statistical analyses were performed using Prism (GraphPad Software Inc., San Diego, CA). All values are expressed as mean ± SEM. Statistical significance was assessed using an unpaired Student’s t test or the Mann-Whitney test for 2-group comparisons. For multiple pairwise comparisons of groups (different genotypes) over time were evaluated by repeated measures 2-way ANOVA with Bonferroni post-hoc tests (corrected p values are given for comparison between genotypes at specific time points). Differences were considered statistically significant at a value of $P<0.05$. 
Supplemental references


Supplemental Figure Legends

Supplemental Figure 1. Generation of mice with endothelial and myeloid cell specific deletion of TRAF6. (A) Schematic depiction of the targeting strategy used for the generation of TRAF6<sub>Floxed</sub> mice. (B) Detection of the TRAF6-deleted allele by PCR on DNA prepared from aortas of ApoE<sup>−/−</sup> or TRAF6<sup>ECiKO/ApoE<sup>−/−</sup> mice that were placed on normal diet or tamoxifen diet and HFD. At the end of the HFD treatment the deleted TRAF6 allele is detected only in the mice that carry the Tie2Cre<sup>ERT2</sup> transgene and that were fed a tamoxifen diet. (C) Immunoblot analysis of TRAF6 expression in endothelial cells isolated from ApoE<sup>−/−</sup> or TRAF6<sup>ECiKO/ApoE<sup>−/−</sup> mice (5 mice were used per condition). (D) Southern blot analysis of EcoRI digested genomic DNA from FACS-sorted naïve peritoneal macrophages isolated from ApoE<sup>−/−</sup> or TRAF6<sup>MYKO/ApoE<sup>−/−</sup> mice (macrophages from 10-12 mice were used per condition). (E) Immunoblot analysis of TRAF6 expression in whole cell extracts from naïve peritoneal macrophages isolated by FACS-assisted sorting from ApoE<sup>−/−</sup> or TRAF6<sup>MYKO/ApoE<sup>−/−</sup> mice. Each lane represents macrophages isolated from an individual mouse.

Supplemental Figure 2. Endothelial-specific ablation of TRAF6 reduces atherosclerosis in female ApoE<sup>−/−</sup> mice. (A-B) Graph showing the quantification of the lesion area of ApoE<sup>−/−</sup> or TRAF6<sup>ECiKO/ApoE<sup>−/−</sup> mice from the two independent groups of mice that were analysed (Mann-Whitney test). Figure 1E shows the pooled results obtained by combining the data from the two independent groups in one graph.

Supplemental Figure 3. The presence of the Tie2CreER<sup>T2</sup> transgene or the tamoxifen treatment does not affect the development of atherosclerosis in ApoE<sup>−/−</sup> mice. (A) Graph showing the body weight of mice after the indicated treatment
and 10 weeks of HFD. (B) Graph showing the quantification of the size of atherosclerotic lesions from ApoE\textsuperscript{−/−} or Tie2CreER\textsuperscript{T2} mice after the indicated treatment and 10 weeks of HFD.

**Supplemental Figure 4.** Myeloid-specific ablation of TRAF6 results in increased atherosclerotic lesion area and necrotic core in ApoE\textsuperscript{−/−} mice. (A-B) Graphs showing the quantification of the lesion area of ApoE\textsuperscript{−/−} or TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{−/−} mice from the two independent groups of mice that were analysed (Mann-Whitney test). Figure 4E shows the pooled results obtained by combining the data from the two independent groups in one graph. (C-D) Graphs showing the quantification of the necrotic core area of ApoE\textsuperscript{−/−} or TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{−/−} mice in each of the two independent groups that were analysed (Mann-Whitney test). Figure 6B shows the pooled results obtained by combining the data from the two independent groups in one graph.

**Supplemental Figure 5.** Gene expression analysis in aortas from female TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{−/−} or ApoE\textsuperscript{−/−} mice shows reduced expression of IL-10 and increased expression of CHOP similarly to male mice. Graphs showing the relative expression of cytokines (A), chemokines (B), adhesion molecules (C) and CHOP (D) in aortas of female TRAF6\textsuperscript{MYKO}/ApoE\textsuperscript{−/−} mice compared to their littermate female ApoE\textsuperscript{−/−} mice (Mann-Whitney test).

**Supplemental Figure 6.** Traf6\textsuperscript{−/−} macrophages do not show altered expression of integrins/adhesion molecules upon oxLDL stimulation. Graphs showing the expression of CD29 and CD11a in bone marrow macrophages after stimulation with oxLDL (50 μg/ml). Data represent the result of an experiment where macrophages from 3 different mice per genotype were used. * represents p<0.05 (two-way ANOVA followed by Bonferroni post-hoc tests).
Supplemental Figure 7. *Traf6*−/− and *MyD88*−/− macrophages show impaired IL-10 expression after oxLDL stimulation. Graph showing the expression of IL-10 in bone marrow macrophages after stimulation with oxLDL (50 μg/ml). Data represent the result of an experiment where macrophages from 4 different mice per genotype were used. *** represents p<0.001 (two-way ANOVA followed by Bonferroni post-hoc tests).
Supplemental Figure 1

A

B

TRAF6 deleted
TRAF6
Cre
ApoE
Tamoxifen + HFD

C

D

E

ApoE−/−  TRAF6εεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεee
Supplemental Figure 6

A

![Graph A: Relative expression of CD29](image)

- $\text{Traf6}^{FL/FL}$
- $\text{Traf6}^{-/-}$

hours (oxLDL)

B

![Graph B: Relative expression of CD11a](image)

- $\text{Traf6}^{FL/FL}$
- $\text{Traf6}^{-/-}$

hours (oxLDL)
Supplemental Figure 7

![Graph showing relative expression of IL-10 over time for different genotypes: Traf6^{FL/FL}, Traf6^{+/−}, and MyD88^{−/−}.](image)

- **Traf6^{FL/FL}**
- **Traf6^{+/−}**
- **MyD88^{−/−}**

**Y-axis:** Relative expression

**X-axis:** Hours (oxLDL)

*** Indicates significant difference.